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Proglucagon is processed to glucagon by prohormone convertase PC2 in αTC1-6 cells

(precursor processing/islets of Langerhans/antisense RNA/α cells)

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ABSTRACT **Proglucagon is processed differentially in the pancreatic α cells and the intestinal L cells to yield either glucagon or glucagon-like peptide 1, respectively, structurally related hormones with opposing metabolic actions. Here, we have studied the processing of proglucagon in αTC1-6 cells, an islet-cell line transformed by simian virus 40 large tumor (T) antigen, a model of the pancreatic α cell. We found that these cells process proglucagon at certain dibasic cleavage sites to release glucagon and only small amounts of glucagon-like peptide 1, as demonstrated by both continuous and pulse–chase labeling experiments. Both normal islet α cells and αTC1-6 cells were shown to express the prohormone convertase PC2 at high levels, but not the related protease PC3. Expression of PC2 antisense RNA in αTC1-6 cells inhibited both PC2 production and proglucagon processing concomitantly. We conclude that PC2 is the key endoprotease responsible for proglucagon processing in cells with the α-cell phenotype.**

Recent studies identified a family of mammalian precursor-processing endopeptidases related to subtilisin and the yeast convertase kexin (1). Two members of this family, PC2 (SPC2) and PC3/PC1 (SPC3), are expressed predominantly in neuroendocrine cells (2, 3) and have been implicated in the processing of proopiomelanocortin, proinsulin, and several other precursors (4–8).

Glucagon is a highly conserved 29-amino acid peptide hormone that is produced mainly in the α cells of the islets of Langerhans (9). It is secreted in response to hypoglycemia and acts in the liver to enhance both glycogenolysis and gluconeogenesis, thus opposing the blood sugar-lowering action of insulin. Glucagon is derived from proglucagon (10), an 18-kDa protein which contains glucagon and two related peptide sequences, glucagon-like peptides 1 and 2 (GLP-1 and -2), bracketed by pairs of basic amino acids (11). The single gene encoding proglucagon is expressed both in the α cells of the islets and in the intestinal L cells, but the proglucagon molecule is processed quite differently in these two cell types. The islet α cells store and release glucagon whereas the intestinal L cells secrete GLP-1-(7–36 amide) (12). The primary role of GLP-1 appears to be the potentiation of insulin secretion from the islet β cell in response to glucose (12). To determine the basis of the highly selective processing of proglucagon which underlies these different tissue-specific secretory patterns, we have studied glucagon biosynthesis in a glucagon-producing/secreting cell line, the αTC1-6 cell line (13, 14). This cell line processes proglucagon in a manner that faithfully reflects the parental pancreatic α cells from which it has been derived. These cells also express high levels of prohormone convertase PC2, and suppression of PC2 levels with antisense RNA results in reduced processing of proglucagon to glucagon. This result is consistent with a role of PC2 as the key processing endoprotease in the biosynthesis of glucagon.

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MATERIALS AND METHODS

Cell Culture. αTC1-6 and βTC3 cells were obtained from S. Efrat (Albert Einstein College of Medicine, New York) and grown as described (13–15).

Antisera. B6/11 glucagon antiserum was obtained from H. Tager (University of Chicago). K4023 antiserum and Glu 001 monoclonal antibody are from Novo-Nordisk (Copenhagen). P7 glucagon antiserum and GPJ3-08 GLP-1 carboxyamidated-C-terminus-specific antiserum were raised in K. Polonsky's laboratory (University of Chicago). The 89-390, 2135 and 165-3 GLP-1 antisera and 8773 GLP-2 antiserum were kindly provided by J. J. Holst and C. Ørskov (Panum Institute, Copenhagen). PC2 pep3 and PC2 pep4 are rabbit PC2 antisera raised against synthetic peptides corresponding to amino acids 586–609 and 611–635, respectively, of both human and mouse PC2.

Metabolic Labeling. αTC1-6 cells were plated in 12-well plates (Costar) at a density of 5×10^5 cells per well. Twenty hours later, the cells were starved for 1 hr in serum-free Dulbecco's modified Eagle's medium (DMEM) lacking methionine, phenylalanine, or leucine singly or in combination and containing 0.25% bovine serum albumin (Sigma). Cells were then incubated at 37°C for 5 or 6 hr in the same medium containing the corresponding amino acid(s) labeled with ^3H or ^{35}S (250 μCi per well; 1 μCi = 37 kBq) (Amersham), and supplemented with Trasylol (aprotinin) at 500 units/ml. The medium was collected, and the cells were washed and suspended in the lysis/immunoprecipitation buffer (LIB) [50 mM Na_2HPO_4, pH 7.4, containing poly(L-lysine) (2.5 μg/ml), bovine serum albumin (1 mg/ml), EDTA (1 mM), phenylmethanesulfonyl fluoride (0.3 mM), Triton X-100 (0.1%), Nonidet P-40 (0.5%), and NaCl, (0.9%)]. A protease inhibitor mixture was freshly added both to the LIB and to the collected medium, to yield 0.1 mM 1,10-phenanthroline, 0.1 mM 3,4 dichloroisocoumarin, 20 μM E64, and 10 μM pepstatin.

For the pulse–chase studies, the labeling medium was removed after 20 min and the cells were washed and incubated in DMEM with 10 μM relevant unlabeled amino acid. At the times indicated, medium and cells were collected and treated as in the steady-labeling experiments.

Immunoprecipitation was performed on cell lysate supernatants or on conditioned media diluted with LIB (1:1), with 1 or 2 μl of antiserum at 4°C overnight. The antigen–antibody complexes were isolated with 10 μl of protein A-agarose beads

Abbreviations: GLP, glucagon-like peptide; GRPP, glicentin-related polypeptide; MPGF, major proglucagon fragment.

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(Pierce), washed three times with LIB, once with TAS buffer (50 mM Tris/100 mM NaCl/0.25% bovine serum albumin, pH 7.6), and analyzed by SDS/PAGE (16) or HPLC.

HPLC. For HPLC analysis, peptides were eluted from the beads with two 100-μl portions of 0.1 M HCl and injected onto a Vydac C_4 reversed-phase HPLC column along with 1 μg of synthetic human glucagon, oxyntomodulin, or GLP-1 (Peninsula Laboratories) as internal standards. The peptides were resolved at 1 ml/min over a three-step gradient of acetonitrile in 0.1 M NaCl/0.01 M HCl (0–20% acetonitrile over 5 min, followed by 20–25% over 5 min, and 25–40% over 60 min). Aliquots (200 μl) of each fraction (1 ml) were assayed for radioactivity in a liquid scintillation counter. The ^3H- and ^{35}S-containing fractions were dried, suspended in loading buffer, and analyzed by SDS/PAGE (16).

Recombinant Plasmid Construction and Transfection Studies. αTC1-6 cells were transfected with pCB6$^+$, an expression vector encoding resistance to neomycin and containing a polylinker site situated between a cytomegalovirus promoter and a human growth hormone polyadenylylation/termination site (17). To construct an antisense PC2 expression vector, pCB6$^+$ was digested with *Eco*RV. A 1-kb mouse PC2 cDNA fragment, encompassing the ribosome binding site and extending 60 bp into the 5′ untranslated region, was amplified by PCR from βTC3 cDNA and subcloned into pCB6$^+$. The orientation was verified by restriction analysis. αTC1-6 cells were transfected by electroporation and selected for resistance to G418 sulfate (0.4 mg/ml; GIBCO-BRL). Individual clones were isolated, grown up, and screened by immunoblot analysis for the amount of PC2 secreted into the medium and present in cell lysates (6, 18). The glucagon-immunoreactive peptides were analyzed on the early-passage cell lysates. Protein (25 μg) was resolved by SDS/PAGE and transferred electrophoretically onto an Immobilon P membrane (Millipore). The gel was stained with Coomassie blue to confirm the effective transfer of the peptides. The blot was developed with the ECL kit (Amersham) using the K4023 antiserum as a primary antibody at a 1:2000 dilution at room temperature.

RESULTS

Identification of Proglucagon Processing Intermediates and Products in αTC1-6 Cells. Proglucagon-derived peptides produced and secreted by αTC1-6 cells were identified by continuous metabolic labeling, immunoprecipitation, chromatographic and electrophoretic characterization, and, for GLP-1, N-terminal radiosequencing.

Fig. 1 shows the resolution by SDS/PAGE of immunoprecipitated proglucagon-derived peptides. Antisera directed against glucagon, GLP-1, and GLP-2 were used. The K4023 antiserum, which recognizes equally well glucagon and its various N- and/or C-terminally extended forms (19), specifically immunoprecipitated five peptides of 19, 9, 7.5, 4.5 and 3.4 kDa (lane 1). The 19-kDa band was identified as proglucagon because its size corresponds well to the calculated molecular mass of 18 kDa (20–24) and because it was bound by glucagon-, GLP-1-, and GLP-2-specific antibodies (Figs. 1 and 2). The 9-kDa band was identified as glicentin (Fig. 3) on the basis of its reactivity with glucagon antiserum. The 7.5- and 3.4-kDa peptides were also immunoprecipitated by the P7 antiserum (lane 2), which recognizes fully processed glucagon. From their sizes the 4.5- and 3.4-kDa peptides can be identified as oxyntomodulin and glucagon, respectively. These assignments were confirmed by HPLC analysis (Fig. 2*A*) using coinjected synthetic human oxyntomodulin and glucagon, respectively, as standards.

Identification of the 9-kDa and 7.5-kDa glucagon-containing intermediates was complicated by the fact that antisera to GLP-1 and GLP-2 also immunoprecipitated a peptide of ≈8 kDa (Fig. 1, lanes 3 and 5). The 7.5-kDa material was eluted

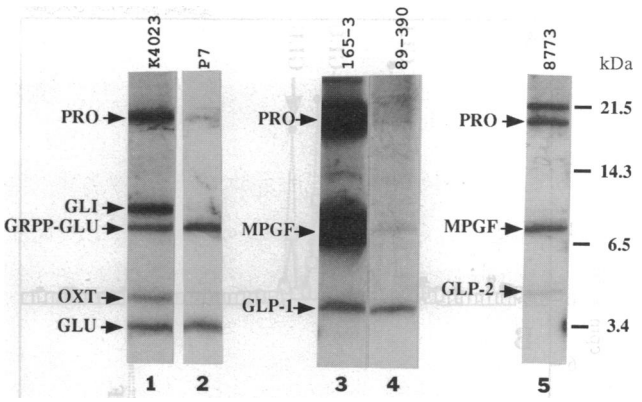

FIG. 1. SDS/PAGE analysis of proglucagon-derived peptides biosynthesized in αTC1-6 cells. αTC1-6 cells were radiolabeled for 6 hr in DMEM containing either [^{35}S]methionine (lanes 1, 2, and 5) or a mixture of [^{35}S]methionine, [^3H]leucine, and [^3H]phenylalanine (lanes 3 and 4). Cell lysates were immunoprecipitated with the antiserum indicated above each lane. Immunoprecipitates were analyzed by SDS/PAGE and fluorography. Migration positions of molecular size markers are indicated. PRO, proglucagon; GLI, glicentin [proglucagon-(1–69)]; GRPP-Glu, proglucagon-(1–61) (GRPP, glicantin-related polypeptide); OXT, oxyntomodulin [proglucagon-(33–69)]; GLU, glucagon [proglucagon-(33–61)]; MPGF, major proglucagon fragment [proglucagon-(72–158)]; GLP-1, GLP-1-(1–36 amide) [proglucagon-(72–107)]; GLP-2, GLP-2-immunoreactive peptide. The 21.5-kDa band shown in lanes 3 and 5 is likely to be nonspecific. It was not immunoprecipitated with glucagon-specific K4023 (lane 1) and GLP-1-specific 2135 (data not shown) antisera, which recognize N- and/or C-terminally extended forms of these peptides. Moreover, it is present in immunoprecipitates from cell lysates but not from conditioned media (data not shown) and is therefore probably not a secreted peptide.

in fraction 35, whereas the ≈8-kDa GLP-1-related material was present in fraction 65 (Fig. 2). Moreover, the molecular mass of these two peptides clearly differed when analyzed in a gel having higher resolution (Fig. 2*C*).

Thus, the 7.5-kDa glucagon-containing peptide can be identified with the proglucagon-(1–61) fragment (GRPP linked to glucagon), since it shares with glucagon the epitope recognized by the P7 antiserum. This epitope includes the C terminus of mature glucagon, since P7 binds glucagon but not oxyntomodulin (Figs. 1 and 2), which differs from glucagon by only a short C-terminal extension (see Fig. 3).

On the other hand, the ≈8-kDa GLP-1-containing peptide was identified as MPGF [proglucagon-(71–158)]¶ because it was immunoprecipitated by GLP-2 C-terminus-specific antiserum 8773 (Fig. 1, lane 5), GLP-1 antiserum 2135 (Fig. 2), and antiserum 165-3 [raised against the N-terminal sequence of GLP-1-(1–37)] (Fig. 1, lane 3), but not by antiserum 89-390 (Fig. 1, lane 4), which is specific for carboxyamidated GLP-1 (see Fig. 3), or by the glucagon antiserum (Fig. 2).

The GLP-1 peptide synthesized by αTC1-6 cells is 4 kDa by SDS/PAGE (Fig. 1, lanes 3 and 4) and binds to both N-terminal-extension-specific antiserum 165-3 and carboxyamidated-specific antisera 89–390 (Fig. 1) and GPJ3-08 (data not shown). It was coeluted with synthetic human GLP-1-(1–36 amide) in two different HPLC systems (Fig. 2). It was therefore identified with GLP-1-(1–36 amide) [proglucagon-(72–107)]. This assignment was confirmed by radiosequenc-

¶The calculated molecular mass of rat MPGF is 10 kDa, in contrast to its apparent size of ≈8 kDa as determined by SDS/PAGE. This discrepancy is not likely to be due to any significant species difference in amino acid sequence, as the overall size of MPGF is very well conserved (20–24), but it could arise from an as yet uncharacterized posttranslational modification (25) which results in a higher mobility of MPGF on SDS/PAGE.

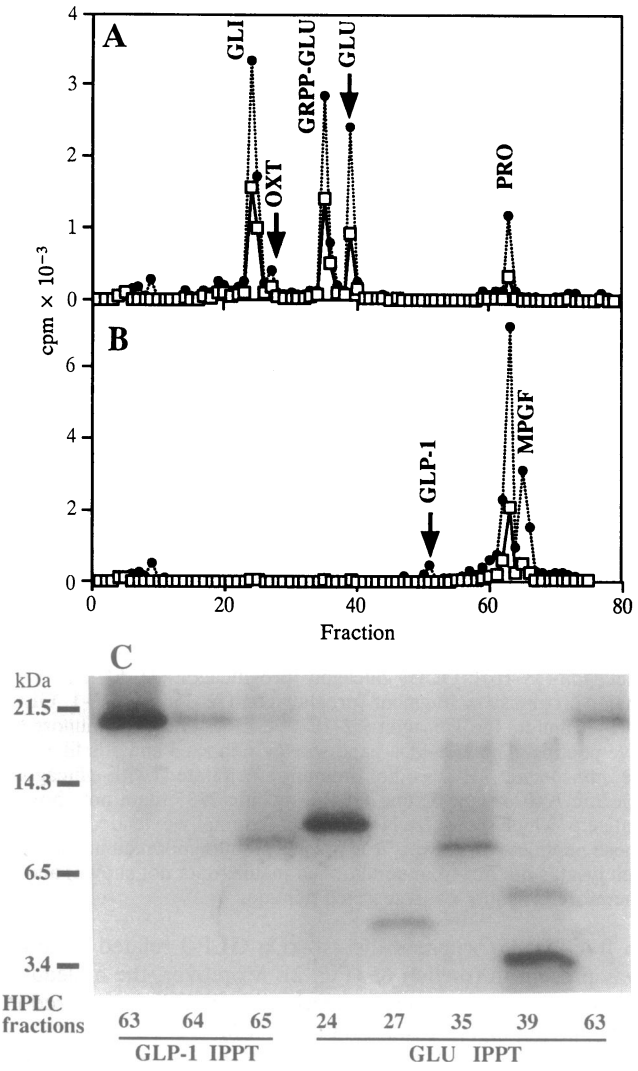

FIG. 2. HPLC analysis of proglucagon-derived peptides secreted by αTC1-6 cells. αTC1-6 cells were radiolabeled for 5 hr in DMEM containing [^{35}S]methionine, [^{3}H]leucine, and [^{3}H]phenylalanine. The medium was immunoprecipitated with anti-glucagon B6/11 (*A*) and anti-GLP-1 2135 (*B*) antisera. Immunoprecipitates (IPPT) were analyzed by HPLC and scintillation counting. Fractions corresponding to the radioactive peaks were analyzed by SDS/PAGE (*C*) to determine the apparent size of each peptide. Elution positions of coinjected synthetic human oxyntomodulin, glucagon, and GLP-1-(1–36 amide) are indicated by arrows. □, ^{35}S; ●, ^{3}H; GLI, glicentin; OXT, oxyntomodulin; GLU, glucagon; PRO, proglucagon.

ing of the [^{3}H]phenylalanine-labeled peptide; no coeluted GLP-1-(7–36 amide) was detected.

The GLP-2-immunoreactive peptide has an apparent molecular mass of 4.7 kDa (Fig. 1, lane 5), higher than the calculated molecular mass of 3.7 kDa for rat GLP-2. This peptide was therefore tentatively identified as an N-terminally extended GLP-2 (see Fig. 3), although the molecular mass of this form calculated from the rat sequence is 5.4 kDa.

Pulse–Chase Studies on Proglucagon Processing in αTC1-6 Cells. The continuous labeling experiments established that proglucagon is cleaved at four different sites during its processing in αTC1-6 cells. To investigate the kinetics of this processing, pulse–chase studies were carried out (Fig. 4). The disappearance of proglucagon was concomitant with the appearance of glicentin and MPGF. These two peptides were first detected after 30 min of chase. No larger transient intermediates were detected. The first event in proglucagon proteolytic processing is therefore the cleavage at the Lys_{70}-Arg_{71} site of the precursor (Fig. 3). The two domains then undergo different fates.

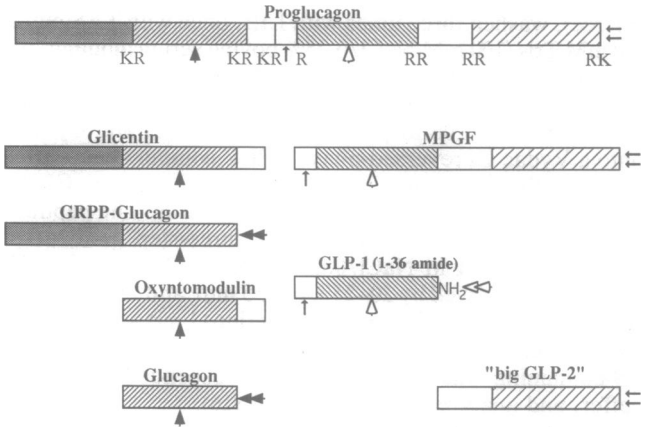

FIG. 3. Schematic representation of proglucagon, processing intermediates, and products identified in αTC1-6 cells. Epitopes of antisera used for immunoprecipitation of the peptides are indicated as follows: solid arrowhead, K4023; double solid arrowhead, P7; open arrowhead, 2135; double open arrowhead, 89-390 and GPJ3-08; small arrow, 165-3; double small arrow, 8773. K, lysine; R, arginine. See legend of Fig. 1 for other abbreviations.

The amount of glicentin increased to reach a peak after 1 hr of chase and then gradually decreased. At the same time, a second wave of intermediates–namely, proglucagon-(1–61) and oxyntomodulin—transiently appeared and gradually disappeared, both with similar kinetics. Glucagon began accumulating after 100 min of chase and was practically the only labeled glucagon-related peptide present in the cell after 6 hr of chase (Fig. 4*A*). Similar pulse–chase data were obtained in an experiment with [^{35}S]methionine labeling. These results indicated that both Lys-Arg processing sites at the N and C termini of the glucagon sequence within the precursor are cleaved at similar rates.

On the other hand, MPGF accumulated as a final processing product and was only partially and slowly cleaved into N-terminally extended GLP-1 (Fig. 4*B*) and presumably "big GLP-2" (Fig. 3). The GLP-1 peptide was first detected after 2 hr of chase. These results are summarized in Fig. 5.

This experiment also suggested that αTC1-6 cells secrete newly synthesized peptides quite rapidly, since approximately half of the precursor was secreted unprocessed and roughly another half of the processing products was also secreted after 6 hr of chase. It is not clear whether the large amount of intact proglucagon in the medium can be accounted for by a high rate of secretion via constitutive pathways or by an elevated basal secretion via regulated secretory pathways. This question was not further investigated.

PC2 Expression in αTC1-6 Cells and Its Function in Proglucagon Processing. The expression in αTC1-6 cells of the neuroendocrine cell-specific prohormone convertases PC2 and PC3 was investigated by Northern blot analysis (Fig. 6). PC2 mRNA was found in large amounts in αTC1-6 cells. On the other hand, we could not detect any signal for PC3 mRNA, even after prolonged exposures. Both RNAs were easily seen in βTC3, an insulin-secreting cell line (15). The pattern found by Northern analysis was confirmed at the protein level by immunoblot analysis of αTC1-6 and βTC3 cell lysates (data not shown). Both PC2 and PC3 have been shown to be involved in proinsulin processing (6). The expression of PC2 in αTC1-6 cells thus suggests that this enzyme might be involved in the α-cell-specific proglucagon-processing phenotype.

To confirm this differential expression of PC2 and PC3 in pancreatic α and β cells, immunohistochemical studies were carried out. Fig. 7 shows a section of an islet from an

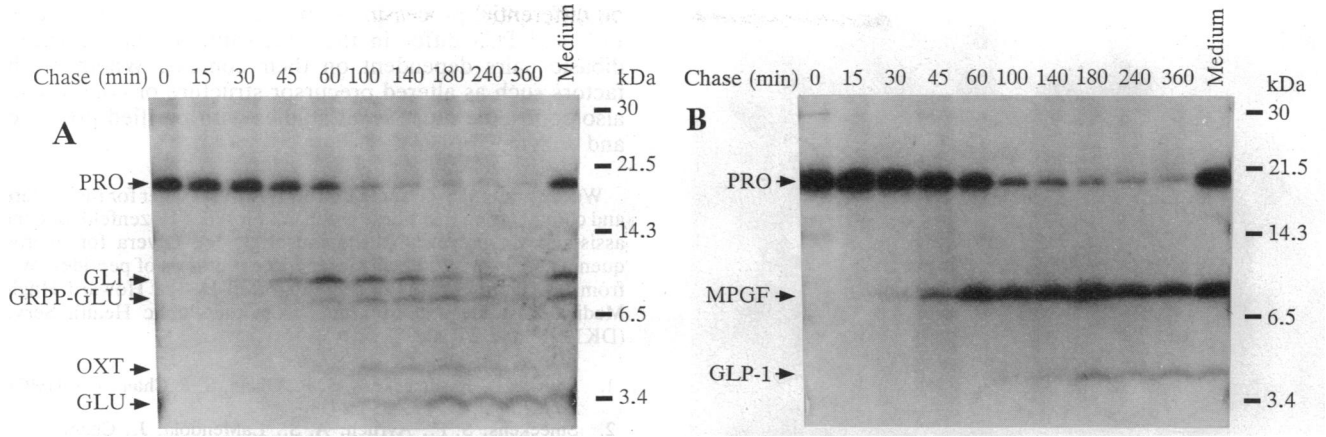

FIG. 4. Pulse–chase study of proglucagon processing in αTC1-6 cells. αTC1-6 cells were incubated for 20 min in DMEM with [^{3}H]phenylalanine and then washed and incubated in medium containing nonradioactive phenylalanine for the time indicated above each lane. Each cell lysate was split into two equal volumes and immunoprecipitated with anti-glucagon (K4023) (*A*) and anti-GLP-1 (2135) (*B*) antisera. The chase medium from the 6-hr incubation was also immunoprecipitated with the same antisera. Immunoprecipitates were analyzed by SDS/PAGE and fluorography. Abbreviations as in the legend to Fig. 1.

alloxan-treated mouse consecutively stained for glucagon and PC2. The loss of β cells due to alloxan emphasizes the α cells, located at the periphery of the islets, which were strongly positive for both glucagon and PC2 but nonreactive for PC3 (data not shown). This pattern was also found with untreated mouse and human islets. β cells were positive for both PC2 and PC3, whereas α cells were reactive only for PC2 (data not shown). PC2 staining was more intense in α cells than in β cells.

To assess the function of PC2 in proglucagon processing, a 1-kb antisense mouse PC2 RNA was expressed in αTC1-6 cells under the control of a cytomegalovirus promoter, to decrease levels of PC2. Four clones were selected which secreted less PC2 into the medium than control cells. The clones were then checked for PC2 levels in cell lysates. PC2 expression was found to be reduced, but was not stable, and rapidly increased to a level comparable to that of untransfected cells by the eighth or ninth passage. We therefore chose to examine the proglucagon processing pattern by immunoblot analysis of earlier passage cell lysates. These results are shown in Fig. 8 in relation to the level of PC2. Two different passages were examined: one with a moderately lowered PC2 level (passage 4 for clones α7, α55, and α63; passage 6 for clone α66) and one with a greater reduction in PC2 (passage 6 for clones α7, α55, and α63; passage 4 for clone α66). For each clone, the moderate reduction in PC2 expression was associated with a processing pattern similar to that found in the untransfected cells, whereas a significant inhibition of processing was seen when PC2 levels were markedly lower. Very little proglucagon-(1–61) intermediate or glucagon could be seen in these instances. Glicentin was also decreased in clones α7, α55, and α66, but not in clone α63. All of these results provide strong support for our conclusion that PC2 participates in the endoproteolytic processing of proglucagon in α cells.

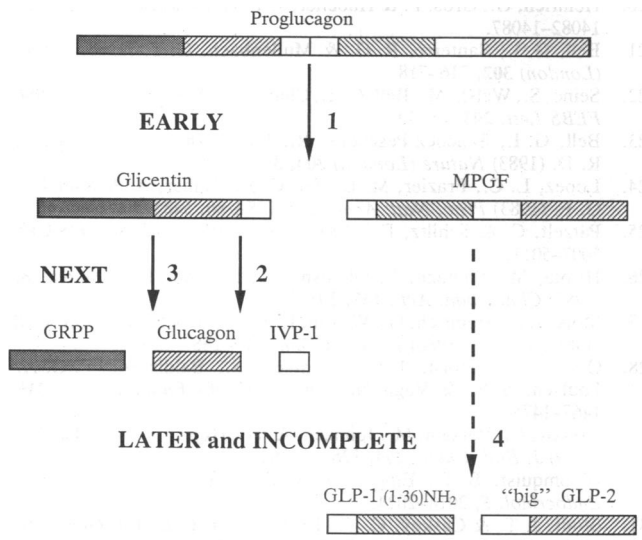

FIG. 5. Proglucagon processing in αTC1-6 cells. Data from Figs. 1–4 are summarized. See text for details.

DISCUSSION

We have examined the pattern of proglucagon processing in glucagon-secreting αTC1-6 cells and shown the close similarity of its processing pattern to that of normal α cells (10, 25–29). We have also shown that this cell line, like pancreatic α cells, expresses the neuroendocrine prohormone convertase PC2, but no detectible PC3. Similarly, by immunocytochemistry we could easily demonstrate expression of PC2, but not PC3, in normal α cells. These results are compatible

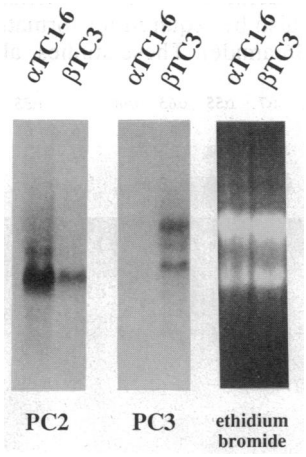

FIG. 6. Northern blot hybridization of the mRNAs encoding processing endopeptidases PC2 and PC3 in two islet-derived cell lines. Twenty micrograms of total RNA from αTC1-6 and βTC3 cells was fractionated in a 1% agarose/formaldehyde gel, blotted onto a nylon membrane and hybridized first with a full-length mouse PC3 cDNA probe, then stripped and rehybridized with a 1-kb mouse PC2 cDNA probe (18). Exposures were 2 hr for the PC2 probe and 16 hr for the PC3 probe.

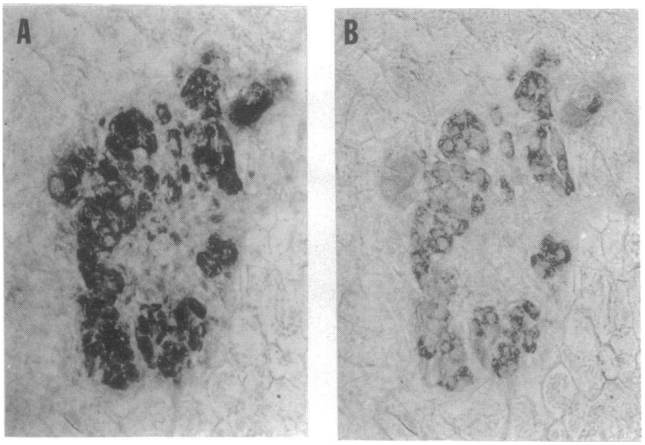

FIG. 7. Glucagon and PC2 colocalization in islets from a mouse treated with alloxan. Pancreas from an alloxan-treated mouse was formalin-fixed and embeded in paraffin. Thin sections (2–4 μm) were incubated with the primary antibody overnight (Glu 001, dilution 1:2000; PC2 pep3, dilution 1:100). The reaction was developed with a biotinylated secondary antibody by using the peroxidase–avidin complex method. The section was first stained for glucagon immunoreactivity (*A*), then destained with absolute ethanol, followed by 2-mercaptoethanol, and restained for PC2 immunoreactivity (*B*). The efficiency of destaining was controlled by developing without antibody. (×400.)

with the hypothesis that PC2 gives rise to the observed processing pattern, and this supposition is supported by the antisense RNA studies. Unlike the experience with AtT20 mouse pituitary tumor cells (30), it has not been possible to achieve stable antisense expression in the αTC1-6 cells, a problem that has been noted in some instances also by others using antisense technology (31).

These results leave unresolved the question of the nature of the convertase(s) that give rise to the intestinal L-cell pattern of processing. Drucker *et al.* (32) have shown that expression of proglucagon in RIN 1046-38 rat insulinoma cells results in extensive processing to both glicentin and glucagon, as well as GLP-1 and -2. Since these cells probably express both PC2 and PC3 at levels comparable to islet β cells (2), it seems probable that PC3 is responsible for most of the additional dibasic cleavages giving rise to the GLPs. However, an as yet unidentified additional monobasic processing enzyme (1) may also be required for formation of the shortened GLP-1-(7–36 amide). These studies, along with others

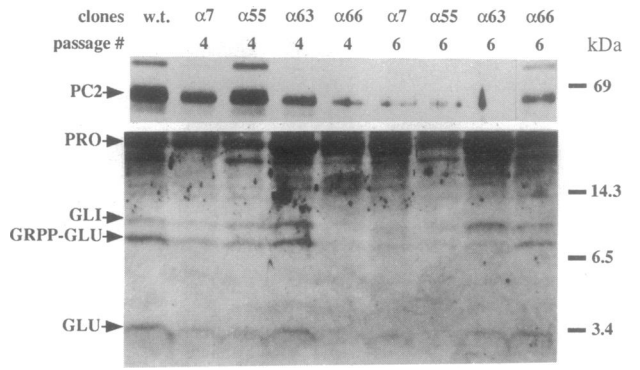

FIG. 8. Inhibition of PC2 synthesis and proglucagon processing by antisense RNA. Individual clones were analyzed by immunoblot both for the expression of PC2 and for proglucagon processing at passages 4 and 6 after transfection with pCB6PC2. (*Upper*) Relative amount of PC2 protein. (*Lower*) Proglucagon processing in the corresponding clones. The clones and passage numbers (after transfection) are indicated above each line; w.t., untransfected αTC1-6 cells. Other abbreviations are as in the legend to Fig. 1.

on differential processing of precursors (4, 5), indicate that PC2 and PC3 differ in their recognition and cleavage of dibasic pairs dependent on their context. Whether other factors such as altered precursor structure or conformation also contribute must await studies with purified precursors and enzymes *in vitro*.

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